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Chromatin Structure of the Ovalbumin Gene Family in the Chicken Oviduct†

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ABSTRACT: We have analyzed the chromosomal structure of the ovalbumin and related X and Y genes in chicken oviduct nuclei. Hen oviduct nuclei were digested with micrococcal nuclease, and the fragmented DNA was separated into four size classes. The concentration of the ovalbumin gene in mono- and dinucleosomal length DNA was 9-13-fold greater than in unfractionated DNA and 20-25-fold greater than in the fraction containing DNA fragments >2500 base pairs. This marked selectivity was not observed with the transcribed X and Y genes nor with transcriptionally inert DNA sequences adjacent to the ovalbumin and X genes. Since the X and Y genes are transcribed at low rates as compared to ovalbumin, these results suggest that micrococcal nuclease can distinguish between genes that exhibit different transcriptional rates. So that a more complete description of the transcriptional domain of the ovalbumin chromatin could be provided, the endogenous nuclease sensitivity of the ovalbumin gene and regions immediately adjacent to this gene was also examined by blot hybridization analysis. The entire coding region of the ovalbumin gene in hen and hormone-treated chick oviduct nuclei was preferentially attacked by this nuclease as compared to the inactive sequences flanking the ovalbumin transcriptional unit and the ovalbumin gene sequences in nuclei from hormone-withdrawn chicks. The endogenous nuclease also introduced a series of site-specific cleavages upstream from the ovalbumin gene in oviduct nuclei from the hen, hormone-stimulated, and hormone-withdrawn chicks. These nuclease cutting sites were not detected in digested nuclei from erythrocytes or kidney. The selective nuclease cutting sites flanking the ovalbumin gene are present, therefore, in oviduct cells prior to the transcriptional activation of the gene whereas the coding region is rendered nuclease sensitive only after hormone treatment.

A model system for the study of transcriptional regulation is the chicken oviduct which synthesizes ovalbumin and other egg white proteins in response to steroid hormones [for a review, see O'Malley et al. (1979)]. The ovalbumin gene exists within a multigene family that is composed of three genes, X, Y, and ovalbumin (Royal et al., 1979; Colbert et al., 1980). These genes, which exhibit limited sequence homology, are expressed in a tissue-specific manner by the oviduct and are coordinately induced by steroid hormones. The maximal rates of transcription of genes X and Y, however, are no more than about 3% and 9%, respectively, of the transcriptional rate of ovalbumin (Colbert et al., 1980; LeMeur et al., 1981). Recent studies using extensive DNase I digestion to examine the chromatin configuration of the ovalbumin gene family have revealed that the ovalbumin, X, and Y genes exist within a contiguous DNase I sensitive domain containing over 100 kilobase(s) (kb) of DNA in hen oviduct chromatin whereas the entire domain is resistant to digestion in spleen, liver, and erythrocyte nuclei (Lawson et al., 1980, 1982). The packaging of the ovalbumin and related X and Y genes in this common

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0006-2960/83/0422-0021$01.50/0

DNase I sensitive conformation in the oviduct is consistent with an earlier report demonstrating that genes that are presumably transcribed at very different rates in this tissue are equally sensitive to DNase I digestion (Garel et al., 1977). The ovalbumin gene (Shepherd et al., 1980), as well as its entire associated domain (Lawson et al., 1982), remains in a DNase I sensitive conformation in the chick oviduct following the transcriptional inactivation of the ovalbumin gene family upon hormone withdrawal, thus providing additional evidence that this chromatin domain is independent of the level of transcription of its included members. Therefore, although the packaging of the ovalbumin, X, and Y genes into this DNase I sensitive domain may provide the structural capability for the coordinate expression of this locus in the oviduct, additional factors must be involved in the modulation of the transcription of the individual genes.

The chicken α- and β-globin gene families and their flanking sequences are also packaged into large domains of moderately DNase I sensitive chromatin in erythroid cells, and the expression of the included genes is related in the sense that their transcription is confined to cells of the erythroid lineage (Stalder et al., 1980a,b). In this case, however, there is a temporal dissociation in the expression of the individual genes within both clusters, which again illustrates that the presence of multigene families in large DNase I sensitive domains is not the exclusive determinant for differential gene transcription. Studies by Weintraub and colleagues have shown that within the framework of these domains exist smaller chromosomal subdomains which are even more sensitive to DNase I than the surrounding regions of chromatin. Striking correlations between the position and temporal appearance of these subdomains and the active transcriptional units of globin genes have been described (Stalder et al., 1980a,b; Weintraub et al., 1981). Studies that have used micrococcal nuclease as a probe for the *Drosophila* heat-shock genes have also shown that the transcriptional units of these genes in chromatin are in a nuclease-sensitive conformation as compared to neighboring nontranscribed regions. In addition, the level of micrococcal nuclease sensitivity of the heat-shock genes is reversible and dependent on their transcriptional activity (Wu et al., 1979; Levy & Noll, 1981). Similarly, micrococcal nuclease digestion studies have revealed alterations in the chromatin structure between the transcriptional units of ribosomal genes and interspersed nontranscribed spacers (Johnson et al., 1979). The sensitivity of the ribosomal transcriptional units is also apparently dynamically related to the expression of these genes during development (Reeves, 1978). These observations provide support for the emerging concept that the larger domains of DNase I sensitive chromatin represent the primary level of organization within which exist subdomains of chromatin structure that are more closely related to the acute transcriptional process.

Micrococcal nuclease also cleaves preferentially the ovalbumin gene in hen oviduct chromatin as compared to the bulk of the DNA and the transcriptionally silent globin genes (Bellard et al., 1978; Bloom & Anderson, 1978a). The ovalbumin gene is similarly attacked preferentially by a nuclease endogenous to oviduct nuclei (Vanderbilt et al., 1982). In contrast to DNase I, however, these nucleases recognize a feature of the ovalbumin chromatin that changes during hormone-induced alterations in the transcription of this gene in the immature chick oviduct (Bloom & Anderson, 1979, 1982; Vanderbilt et al., 1982). There is a progressive decrease in the sensitivity of the ovalbumin chromatin to these nucleases following estrogen withdrawal, and this specific chromatin region is rapidly reorganized into a nuclease-sensitive conformation after administration of estrogen or progesterone. These nucleases, therefore, recognize a feature of the ovalbumin chromatin that is apparently related to the expression of the ovalbumin gene and hence may be able to distinguish the active ovalbumin transcriptional unit from inactive flanking sequences in the oviduct. In the first part of this study, we examined this possibility by studying the micrococcal nuclease sensitivity of the ovalbumin gene family and flanking sequences in hen oviduct nuclei. We then used the endogenous nuclease to provide a description, in terms of nuclease sensitivity, of the chromatin subdomain surrounding the ovalbumin transcriptional unit in the chicken oviduct.

Experimental Procedures

Preparation and Digestion of Nuclei. Four-day-old female chicks were injected with 2.5 mg/day of diethylstilbestrol for 14 days (primary estrogen stimulation) and then withdrawn from the hormone for 4–5 days. Withdrawn chicks received a single injection of diethylstilbestrol (4 mg) plus progesterone (4 mg) 12 h before they were sacrificed (secondary hormone stimulation). All laying hens were injected with a mixture of these two hormones (25 mg of each) 12 h before they were killed. Oviduct nuclei were prepared as described by Bloom & Anderson (1978b), and erythrocyte nuclei were prepared according to the method of Weintraub & Groudine (1976). In the experiments described in Figures 4–8, the above procedures were employed except that the centrifugation of oviduct nuclei through 1.5 M sucrose was omitted and all centrifugation steps were for 2 min. Nuclei were washed in digestion buffer I [0.35 M sucrose, 25 mM KCl, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4] or digestion buffer II (0.35 M sucrose, 25 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 10 mM Tris-HCl, pH 7.4) and resuspended in the same buffer at a concentration of 40 A_{260} units/mL. Nuclei in buffer I were incubated with micrococcal nuclease (50 units/mL) for 5 min at 37 °C or were autodigested in buffer II at 37 °C for the times indicated in the figure legends.

Preparation and Electrophoretic Analysis of DNA. Nuclei were made 1 M NaCl/1% sodium dodecyl sulfate (NaDodSO_4) and deproteinized by extractions with chloroform/isoamyl alcohol (24:1). The DNA was ethanol precipitated and dissolved in 5 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.0). For solution hybridization studies, DNA from undigested nuclei or from the digested samples indicated in the figure legends was sonicated by using six 20-s bursts with intermittent cooling to an average single-stranded length of 400 nucleotides as determined by denaturing alkaline agarose gel electrophoresis. Following sonication, samples were made 0.3 N NaOH/0.1% NaDodSO_4 and incubated at 37 °C for 18 h to hydrolyze the RNA. Samples were then neutralized, extracted, ethanol precipitated, and dissolved in 5 mM EDTA. For blot hybridization analysis, the DNA was treated with pancreatic RNase (50 μg/mL for 1 h at 37 °C), chloroform extracted, ethanol precipitated twice, and dissolved in H_2O. Digestion with *Hin*dIII, *Eco*RI, and *Ava*II (Bethesda Research Laboratories) was carried out in buffers recommended by the manufacturer for 4 h at 37 °C at 6–8 units of enzyme/μg of chicken DNA.

Native DNA samples were analyzed on 1.1% or 1.7% agarose slab gels containing 40 mM Tris-HCl (pH 7.8), 20 mM NaOAc, and 2 mM EDTA. Gels were stained in 1 μg/mL ethidium bromide for 30 min, visualized with a long-wave transilluminator, and immediately photographed.

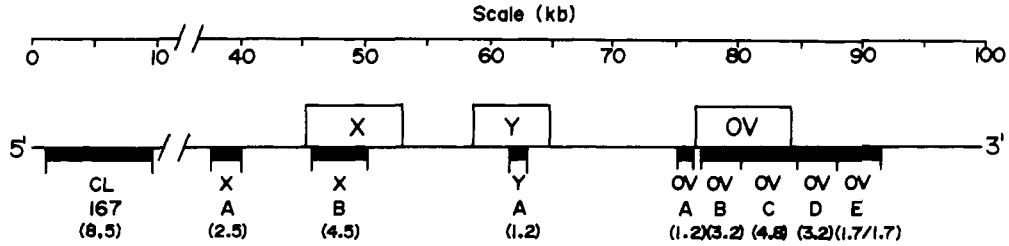

FIGURE 1: Map of the ovalbumin gene family in chicken DNA. The cloned restriction fragments indicated by the dark bars below the map were used as hybridization probes. Their lengths, in parentheses, are given in kilobases. The fragments are identified in this paper by designating the gene region within the cluster followed by a letter corresponding to that portion of the gene region shown in the figure. Fragments X A, X B, Y A, and OV A–E were cloned and isolated as described previously (Colbert et al., 1980; Lawson et al., 1980; Roop et al., 1980). The fragment designated CL 167 was prepared as described by Lawson et al. (1982). OV E, which is specific for the 3.4-kb region shown in the figure, is composed of two adjacent 1.7-kb fragments (Lawson et al., 1980).

For preparative gel electrophoresis, 550 mg of DNA from micrococcal nuclease treated nuclei was fractionated into four molecular weight classes on 1.4% agarose gel slabs (15–20 mg of DNA/slab) as described previously (Bloom & Anderson, 1978a, 1979).

Hybridization. The preparation and characterization of the clones used in these studies have been described previously (Colbert et al., 1980; Lawson et al., 1980, 1982; Roop et al., 1980). Purified cloned DNA was digested with the appropriate restriction endonuclease(s), and the desired fragments were isolated by agarose gel electrophoresis (Roop et al., 1980). Isolated restriction fragments were labeled by nick translation (Nick Translation Kits from New England Nuclear) in the presence of [^{32}P]dCTP (400–700 Ci/mmol). The reaction mixtures were made 200 mM EDTA and heated to 68 °C for 3 min, and the nick-translated probes [$(1–2) \times 10^8$ cpm/μg] or ^{32}P-labeled globin cDNA (Bloom & Anderson, 1978a) was recovered from the excluded fractions following gel filtration over Sephadex G-100. Solution hybridizations were performed as described in the legend to Figure 3 and detailed previously (Bloom & Anderson, 1978a). Transfer of DNA from agarose gels to nitrocellulose filters (Schleicher & Schuell, BA85) and hybridization of the ^{32}P-labeled probes to the immobilized DNA were performed by using the procedure of Southern (1975) according to conditions described elsewhere (Vanderbilt et al., 1982). In these experiments, 4 μg of DNA was electrophoresed on each agarose gel lane, and the dry nitrocellulose filters were exposed to Kodak XAR-5 film backed by one intensifying screen for 4–14 days. The DNA fragment lengths were determined by reference to *Hae*III fragments of ϕX174 and *Hin*dIII fragments of phage λ.

Electrophoresis of the DNA samples in Figure 4 on a 0.8% agarose gel (20 μg/lane) revealed that the nondigested oviduct DNA was shorter than the nondigested erythrocyte DNA. Mechanical shearing of the oviduct DNA during deproteinization was probably responsible for this difference since the oviduct DNA in this experiment was extracted with chloroform 5 times whereas the erythrocyte DNA was extracted twice. This effect was presumably responsible for the somewhat weaker hybridization signals exhibited by the DNA from the nonincubated oviduct as compared to erythrocyte nuclei with probes OV A–E as well as with the globin cDNA (Figure 5, zero-time incubations). In support of this interpretation, when nonincubated erythrocyte and oviduct nuclei were each extracted 3 times, the size of the bulk of the DNA and the intensities of the hybridization signals were essentially the same (see Figures 6 and 7, zero-time incubations).

Results

Sensitivity of the Ovalbumin Gene Family to Micrococcal Nuclease in Hen Oviduct Nuclei. A map of the ovalbumin gene family, indicating the relative positions and lengths of the cloned fragments used in the present study, is shown in Figure 1. The individual restriction fragments were isolated, labeled by nick translation, and used to detect their respective sequences in chicken DNA as described below. The restriction fragments are identified in the text and figures by designating the gene region within the cluster (X, Y, or OV) and by a letter (A, B, C, D, or E) corresponding to the portions of the gene region shown in Figure 1. The fragment designated CL 167, which is located about 40 kb upstream from the X gene, lies outside of the DNase I sensitive domain in hen oviduct chromatin. All other fragments shown in the figure correspond to sequences in oviduct chromatin that are selectively destroyed by extensive DNase I digestion (Lawson et al., 1980, 1982).

Previous correlations between the hormone-induced expression of the ovalbumin gene and its chromatin sensitivity to micrococcal nuclease in the chick oviduct imply that the micrococcal nuclease sensitive feature of the ovalbumin chromatin is related to the transcriptional rate of the ovalbumin gene (Bloom & Anderson, 1979, 1982). To determine if this enzyme can distinguish between chromatin regions that are transcribed at different rates in the laying hen oviduct, we have examined the nuclease sensitivity of the ovalbumin, X, and Y genes and the transcriptionally inactive DNA sequences flanking these genes. Hen oviduct nuclei were digested with micrococcal nuclease until about 1% of the DNA was converted to mononucleosomal length fragments. The DNA extracted from these nuclei was separated into four size classes by preparative agarose gel electrophoresis. Samples of the resulting DNA size classes are displayed on the analytical agarose gel in Figure 2. In Figure 3, increasing amounts of DNA from each size class along with nondigested DNA were annealed to trace amounts of nick-translated restriction fragment probes that correspond to the coding regions for ovalbumin (OV B and OV C), Y (Y A), and X (X B) and to nontranscribed DNA at the 3′ side of the ovalbumin gene (OV E) and at the 5′ side of the X gene (X A and CL 167). The $C_0t_{1/2}$ values for these and other hybridization reactions and the relative concentrations of specific DNA sequences in the four size classes from three separate experiments are given in Table I. The concentrations of transcribed ovalbumin gene sequences in mono- and dinucleosomal length fragments (size class 1) were 9–13-fold greater than in unfractionated DNA and 20–25-fold greater than in the largest molecular weight DNA (size class 4). Both the 5′ (OV B) and 3′ (OV C) regions of the ovalbumin coding sequence were distributed similarly in the four DNA size classes, indicating that the entire coding region of the ovalbumin chromatin is sensitive to micrococcal nuclease. This marked selectivity, however, was not observed with the nontranscribed sequences immediately adjacent to the ovalbumin gene at both the 5′ (OV A)

Table I: Concentration of DNA Sequences in Different Size DNA Fragments

hybridization probe[a]	$C_0t_{1/2}$ value for DNA size class					rel concn[c] for DNA size class				
	U[b]	1	2	3	4	U	1	2	3	4
CL 167[d]	2000	2000	2000	2000	2000	1.0	1.0	1.0	1.0	1.0
X A[d]	3500	2200	2200	2200	3500	1.0	1.6	1.6	1.6	1.0
X B[d]	5000	5000	5000	5000	5000	1.0	1.0	1.0	1.0	1.0
X B	4900	3500	3500	3500	4900	1.0	1.4	1.4	1.4	1.0
Y A[d]	3000	3000	3000	3000	3000	1.0	1.0	1.0	1.0	1.0
Y A	4400	4400	4400	4400	4400	1.0	1.0	1.0	1.0	1.0
OV A	3100	2200	1900	3000	3400	1.0	1.4	1.6	1.0	0.91
OV B[d]	5800	450	1000	3200	13000	1.0	12.8	5.8	1.8	0.45
OV B	3000	350	780	1200	6000	1.0	8.6	3.8	2.5	0.50
OV C[d]	5000	450	1100	2800	12000	1.0	11.1	4.5	1.8	0.42
OV C	3200	310	730	2400	7000	1.0	10.3	4.4	1.4	0.46
OV D	2800	2800	2800	2800	2800	1.0	1.0	1.0	1.0	1.0
OV E[d]	2500	2500	2500	2500	2500	1.0	1.0	1.0	1.0	1.0

[a] The location of these restriction fragments is shown in Figure 1. [b] Unfractionated nondigested DNA. [c] Concentration relative to unfractionated nondigested DNA as determined by the $C_0t_{1/2}$ values. [d] $C_0t_{1/2}$ values were determined from the data in Figure 3 as described by Wetmur & Davidson (1968). All other $C_0t_{1/2}$ values were determined from hybridization analyses (not shown) similar to those shown in Figure 3.

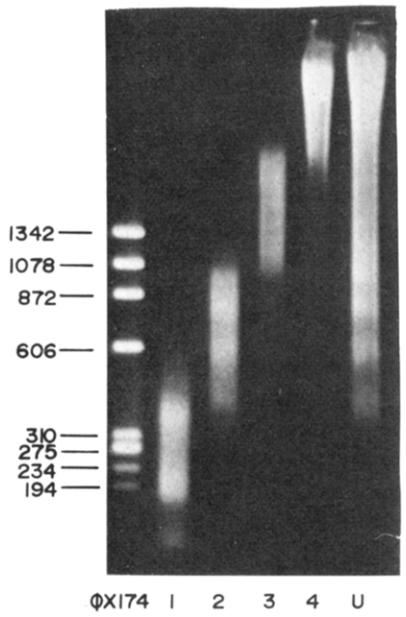

FIGURE 2: Electrophoretic analysis of the four DNA size classes. Nuclei from 600 g of hen oviduct were incubated with micrococcal nuclease (50 units/mL) for 5 min. DNA was extracted from the digested nuclei and separated into four molecular weight classes by preparative agarose gel electrophoresis. (Lane U) Unfractionated DNA before separation by preparative gel electrophoresis analyzed on a 1.7% agarose gel; (lanes 1–4) four DNA size classes in order of increasing molecular weight; (lane ϕX174) DNA fragments from a *Hae*III digest of bacteriophage ϕX174. In three separate experiments, the size classes 1, 2, 3, and 4, which corresponded to DNA fragment lengths of 100–400, 400–1000, 1000–2500, and greater than 2500 bp, respectively, contained 2, 3, 12, and 83% of the DNA, respectively.

and 3′ (OV D) ends, suggesting that the enhanced sensitivity does not extend far beyond the ends of the coding region. Similarly, enhanced nuclease sensitivity was not detected with the transcriptionally inert DNA sequences upstream from the X gene (X A) or with the X (X B) and Y (Y A) genes themselves which are transcribed at low rates as compared to ovalbumin in the hen oviduct (Colbert et al., 1980; LeMeur et al., 1981). The heightened nuclease sensitivity of the ovalbumin gene and the lack of detectable sensitivity of the X and Y genes provide additional support for the proposal that micrococcal nuclease can distinguish between genes that exhibit different transcriptional rates in oviduct chromatin and that the property recognized selectively by this enzyme is related to very rapid transcription at the chromatin locus. In addition, since the nontranscribed sequences corresponding to OV A, OV D, OV E, and X A, as well as genes X and Y, exhibited a DNase I sensitivity indistinguishable from that observed for transcribed ovalbumin regions (Lawson et al., 1980, 1982), the results in Figure 3 and Table I confirm that micrococcal nuclease and DNase I can recognize different structural parameters of active chromatin and that the recognition sites for micrococcal nuclease are more closely related to transcription.

Endogenous Nuclease Sensitivity of the Ovalbumin Coding DNA. The endogenous nuclease associated with purified nuclei from a variety of avian and mammalian tissues is similar to micrococcal nuclease in that it attacks linker DNA between nucleosomes, producing a 200-base-pair (bp) periodicity early in the digestion (Burgoyne & Hewish, 1978; Vanderbilt et al., 1982). This nuclease, like micrococcal nuclease, apparently recognizes a feature of the ovalbumin chromatin that is transcription specific since the transcribed ovalbumin gene in hen and steroid-treated chick oviduct nuclei is cleaved selectively by this enzyme as compared to the inactive ovalbumin gene in oviduct nuclei from hormone-withdrawn chicks (Vanderbilt et al., 1982). It was of interest, therefore, to determine whether the endogenous nuclease can distinguish between the ovalbumin coding DNA and the transcriptionally inactive flanking sequences in hen oviduct nuclei. To evaluate this possibility and to provide a more complete description of the nuclease-sensitive conformation of the ovalbumin chromatin, we have examined the endogenous nuclease sensitivity of the ovalbumin gene and regions immediately adjacent to this gene by blot hybridization analysis. Hen oviduct and erythrocyte nuclei were incubated for up to 1 h at 37 °C in the absence of exogenous nucleases. Following incubation, samples of the DNA were electrophoresed on an agarose gel and stained (Figure 4). The progressive decrease in the molecular weight of the DNA with increasing incubation time resulted from the action of endogenous nuclease in these nuclei. Samples of this DNA were treated with *Hin*dIII, *Eco*RI, or *Ava*II, electrophoresed on agarose gels, blotted, and probed with various nick-translated DNA fragments (OV A–OV E) or globin cDNA (Figure 5). The results show that the bands containing the coding regions of the ovalbumin gene (OV B and OV C) are preferentially lost in oviduct nuclei during in vitro incubation as compared to these sequences in erythrocyte nuclei and as compared to the globin genes in oviduct nuclei which are transcriptionally silent. A preferential reduction

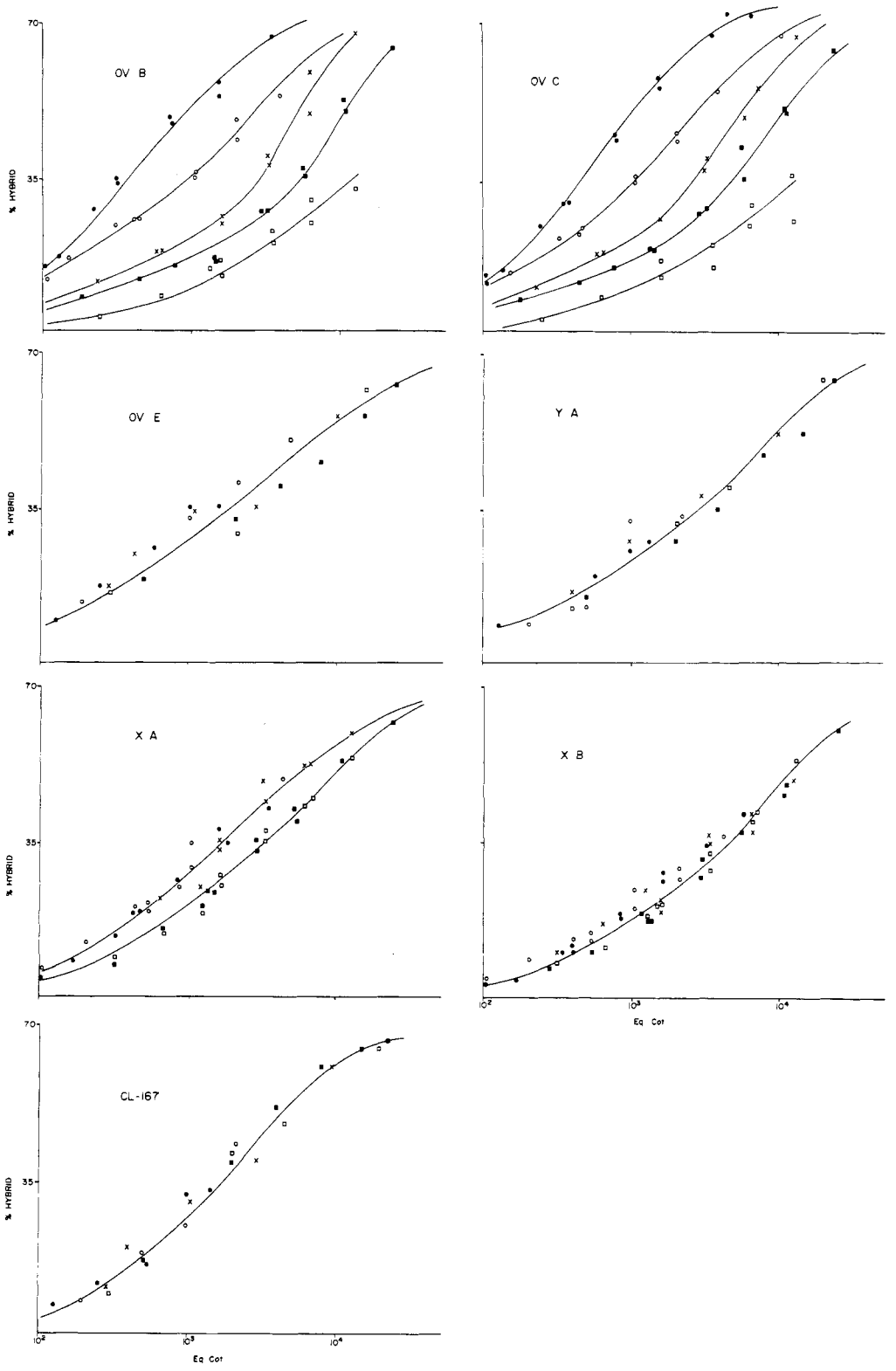

FIGURE 3: Distribution of specific sequences in different size DNA fragments. Hen oviduct nuclei were digested with micrococcal nuclease, and the fragmented DNA was separated into four size classes as described in Figure 2. The three larger molecular weight classes of DNA and nondigested DNA were sonicated to average single-strand lengths of 400 nucleotides, and all DNA samples were then subjected to base hydrolysis to remove RNA. DNA (0.01–11 mg/mL) from size classes 1 (●), 2 (○), 3 (×), and 4 (□) and nondigested DNA (■) were annealed in a volume of 50 μL to the indicated ^{32}P-labeled nick-translated probes [(1.5–2) × 10^4 cpm/mL] for 24 h at 68 °C, and hybrid formation was assayed by resistance to S1 nuclease. The self-annealing of the probes was monitored by parallel hybridizations in which the chicken DNA was replaced by salmon sperm DNA. These values, which rarely exceeded 10% of the total S1 resistant radioactivity, have been subtracted from the data.

in the intensity of both the 5′ (OV B) and 3′ (OV C) coding regions of the ovalbumin gene in oviduct DNA was clearly apparent after 20 min of nuclear incubation. In contrast, transcriptionally inactive sequences flanking both the 5′ end (OV A) and the 3′ end (OV D and OV E) of the ovalbumin transcriptional unit appear relatively insensitive to endogenous nuclease digestion.

An earlier study demonstrated that the 3′ region of the

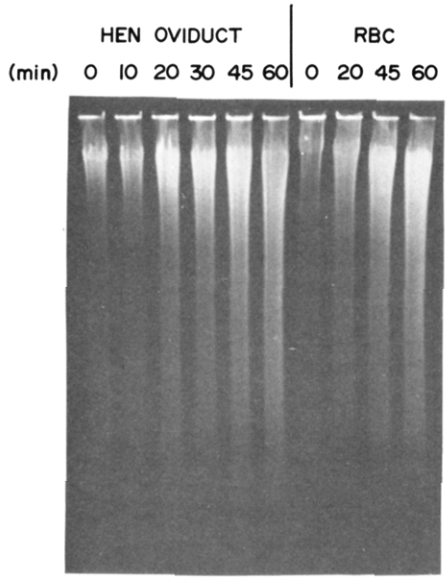

FIGURE 4: Digestion of nuclear DNA by the endogenous nuclease. Nuclei from hen oviducts and erythrocytes were incubated at 37 °C for the indicated times. The purified DNA was electrophoresed on a 1.1% agarose gel and stained with ethidium bromide.

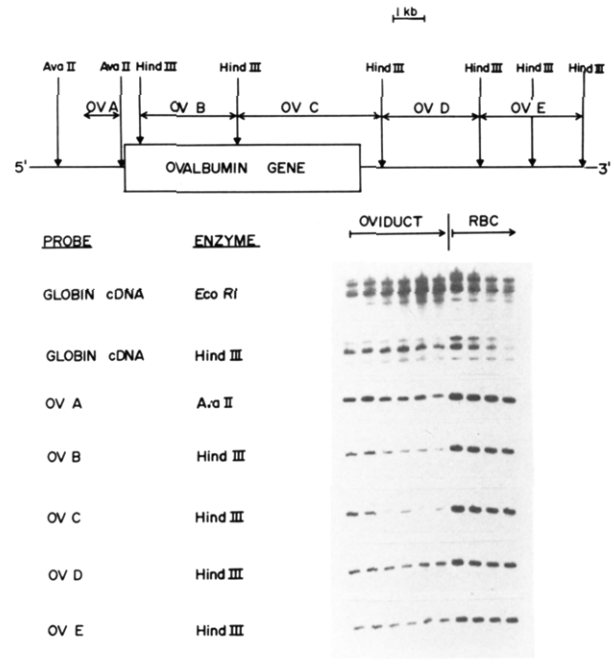

FIGURE 5: Endogenous nuclease sensitivity of the ovalbumin gene and nontranscribed flanking sequences in hen oviduct and erythrocyte nuclei. The map at the top of the figure shows the location of relevant restriction sites in the ovalbumin gene and flanking sequences in chicken DNA and the restriction fragment probes (OV A–OV E) that were used in these experiments (see references in Figure 1 for further details). DNA samples from the experiment described in Figure 4 were digested to completion with *Eco*RI, *Hin*dIII, or *Ava*II, electrophoresed on 1.1% agarose gels, and transferred to nitrocellulose filters. The filters were hybridized to ^{32}P-labeled globin cDNA or to the ^{32}P-labeled nick-translated probes OV A–OV E. The horizontal arrows above the autoradiograms indicate increasing endogenous nuclease digestion time (see Figure 4). Only the portions of the resulting autoradiograms that contained major bands are presented in the figure. The lengths of the fragments detected with probes OV A–OV E were 2.0, 3.2, 4.8, 3.2, and 1.7 kb, respectively. The lengths of the fragments that reacted with globin cDNA ranged from 4.6 to 9.1 kb. All bands were slightly more intense in erythrocyte than in oviduct zero-time DNA samples presumably because of mechanical shearing of the oviduct DNA during deproteinization (see Experimental Procedures). Optical density scans of the autoradiograms (not shown) confirmed that the bands corresponding to the ovalbumin coding sequences (OV B and OV C) were lost more rapidly in oviduct nuclei as compared to these sequences in erythrocyte nuclei, the globin sequences in oviduct nuclei, and ovalbumin gene flanking sequences (OV A, OV D, and OV E) in oviduct and erythrocyte nuclei.

ovalbumin transcriptional unit was selectively attacked by the endogenous nuclease in oviduct nuclei from hormone-stimulated but not hormone-withdrawn chicks (Vanderbilt et al., 1982). To determine if the entire coding region was similarly affected by hormone-induced transcription, we examined the endogenous nuclease sensitivity of both the 5′ and 3′ regions of the ovalbumin chromatin in oviduct nuclei from chicks in various hormonal states (Figure 6). Nuclei from hen erythrocytes (A), primary estrogen-stimulated chick oviducts (B), estrogen-withdrawn oviducts (C), and secondary hormone-stimulated oviducts (D) were incubated at 37 °C for the indicated times. Following incubation, DNA was digested with *Hin*dIII, electrophoresed, blotted, and probed with nick-translated fragments corresponding to the 5′ (OV B) and 3′ (OV C) regions of the ovalbumin gene. The results indicate that both regions of the gene are digested at a faster rate when the gene is actively transcribed in the hormone-treated oviducts as compared to when the gene is quiescent in the erythrocyte and hormone-withdrawn oviducts. It appears, therefore, that the entire coding region responds to hormonal activation in a rather uniform manner.

Endogenous Nuclease Sensitivity of Ovalbumin Flanking Sequences. The results presented in Table I and Figure 5 indicate that the entire coding region of the active ovalbumin gene in oviduct chromatin is sensitive to micrococcal nuclease and the endogenous nuclease whereas the flanking DNA is resistant to digestion by these enzymes. There appears, then, to be a transition in chromatin structure at the ends of the transcriptional unit of the ovalbumin gene. The existence of a chromatin boundary in the vicinity of the 3′ end of the transcribing ovalbumin gene is further supported by the studies shown in Figure 7. As shown in the map at the top of Figure 7, there is an internal *Eco*RI site at 2.1 kb before the 3′ terminus and another 7.1 kb downstream. As predicted from this map, DNA obtained from nuclei that were not digested with the endogenous nuclease yielded a single band of 9.2 kb when digested with *Eco*RI and then hybridized to the 3′ flanking probe OV D (see Figure 7, zero-time incubations). If the endogenous nuclease cleaves only the coding regions of the active ovalbumin gene and not the flanking DNA at the 3′ side as the results in Figure 5 suggest, then this 9.2-kb parent band should be shortened in the digested oviduct nuclei by 2.1 kb to give rise to a 7.1-kb subband containing only 3′ flanking sequences. As shown in Figure 7 (left), a discrete subband of about 7.1 kb was indeed produced at the expense of the 9.2-kb parent fragment in the digested hen oviduct nuclei. This subband was not observed in the digested DNA from erythrocyte nuclei (Figure 7, left), nor was it observed when the DNA from digested hen oviduct was not restricted with *Eco*RI (data not shown). The active conformation, therefore, apparently terminates at a site that is roughly coincident with the 3′ end of the gene (Roop et al., 1980; Tsai et al., 1980). In addition, as shown in the right panel of Figure 7, this 7.1-kb subband was generated in digested oviduct nuclei from secondary hormone-stimulated chicks but not in oviduct nuclei obtained from chicks withdrawn from estrogen. The transition from sensitive to resistant chromatin at the 3′ end of the ovalbumin gene is therefore correlated with the gene's transcriptional activity in the oviduct.

Endogenous Nuclease Cutting Sites Upstream from the Ovalbumin Gene. Hypersensitive DNase I cutting sites up-

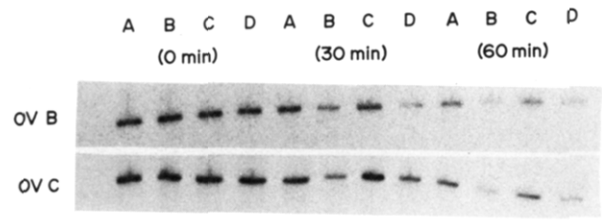

FIGURE 6: Effects of hormones on the endogenous nuclease sensitivity of the ovalbumin gene in chick oviduct nuclei. Nuclei from chick erythrocytes (A), primary estrogen-stimulated chick oviducts (B), estrogen-withdrawn oviducts (C), and secondary hormone-stimulated oviducts (D) were incubated for the indicated times. Purified DNA was digested with *Hin*dIII, electrophoresed on 1.1% agarose gels, blotted onto nitrocellulose sheets, and hybridized to ^{32}P-labeled nick-translated probes OV B or OV C (Figures 1 and 5). Only the portion of the resulting autoradiograms that contained bands is shown. The rates of digestion of the total nuclear DNA by the endogenous nuclease were similar in nuclei from the four tissues as revealed by electrophoretic analysis of the DNA samples prior to treatment with *Hin*dIII (data not shown).

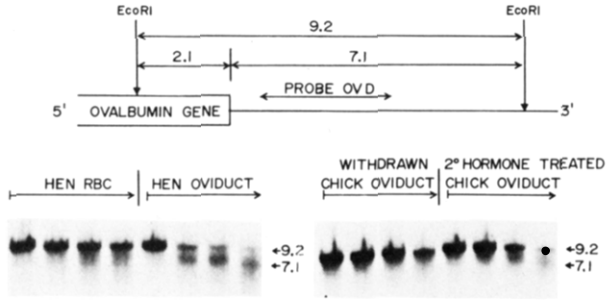

FIGURE 7: Endogenous nuclease sensitivity at the 3′ end of the ovalbumin gene. The map at the top of the figure shows the location of the relevant *Eco*RI sites and probe OV D. (Left) Hen erythrocyte and oviduct nuclei were incubated at 37 °C for 0, 20, 45, and 60 min. The horizontal arrows above the autoradiogram indicate increasing digestion time. The DNA was digested with *Eco*RI, electrophoresed on a 1.1% agarose gel, blotted, and probed with OV D. Only the portion of the resulting autoradiogram that contained bands is shown. (Right) Oviduct nuclei from estrogen-withdrawn and secondary hormone-stimulated chicks were incubated for 0, 5, 15, and 30 min, and the purified DNA was treated as described above. The rates of digestion of total DNA in the nuclei from these two sources were similar (data not shown).

stream from the 5′ side of a variety of genes in chromatin have been described [for a review, see Elgin (1981)]. However, attempts to demonstrate specific DNase I cutting sites upstream from the ovalbumin gene in oviduct chromatin have thus far been unsuccessful (Kuo et al., 1979; Bellard et al., 1980). As shown in Figure 8, such sites are readily detected by the mapping procedure of Wu (1980) in oviduct nuclei digested with the endogenous nuclease. In Figure 8A, hen oviduct and erythrocyte nuclei were incubated for up to 1 h at 37 °C. Following incubation, the nuclear DNA was purified, restricted with *Eco*RI, blotted, and hybridized to probe OV A. As predicted from the map at the top of the figure, DNA obtained from hen oviduct or erythrocyte nuclei that were not incubated in vitro yielded one band of 13.6 kb when digested with *Eco*RI and hybridized to probe OV A. A series of at least seven discrete subfragments appear in the oviduct DNA during digestion of nuclei with the endogenous nuclease. These subbands are apparently cell-type specific since they were not generated during the in vitro incubation of erythrocyte nuclei (Figure 8A). Similarly, these subfragments were not produced in kidney nuclei nor were they detected in erythrocyte nuclei digested for longer periods of time than those shown in Figure 8A (data not shown). Since these subfragments were not observed when the digested oviduct DNA was not re-

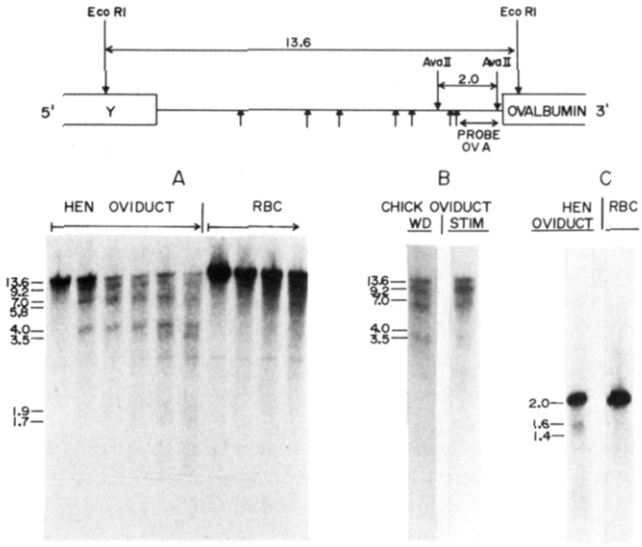

FIGURE 8: Specific endogenous nuclease cutting sites upstream from the ovalbumin gene in oviduct nuclei. The map at the top of the figure shows the location of the relevant *Eco*RI and *Ava*II sites and probe OV A. The blunt arrows indicate the points of endogenous nuclease cutting sites in oviduct nuclei mapped in this study. (A) Hen oviduct and erythrocyte DNA samples from the experiment described in Figure 4 were restricted with *Eco*RI, electrophoresed on a 1.1% agarose gel, blotted, and probed with OV A. The numbers on the left refer to the length of the fragments in kilobases. The arrows above the autoradiogram indicate increasing digestion time (see Figure 4). (B) Oviduct nuclei from estrogen-withdrawn (WD) and secondary hormone-stimulated (STIM) chicks were incubated for 30 min as described in Figure 7. The purified DNA was then treated as described in (A). (C) Nuclei from hen oviducts and erythrocytes were incubated for 45 min. The purified DNA was treated with *Ava*II, electrophoresed, blotted, and probed with OV A. The two minor subbands were detected in oviduct but not erythrocyte DNA at 30, 45, and 60 min of nuclear incubation. These bands, which are not shown in Figure 5, were the only subbands detected in the experiments described in Figure 5.

stricted with *Eco*RI (data not shown), we surmise that one end of each band was produced from the *Eco*RI cleavage near the 5′ end of the ovalbumin gene while the other arose from a specific endogenous nuclease cut upstream. From this preliminary analysis, the endogenous nuclease cuts can be mapped to positions that are about 8.8, 6.6, 5.4, 3.6, 3.1, 1.5, and 1.3 kb upstream from the 5′ end of the ovalbumin gene. The hypersensitive sites positioned at 8.8, 6.6, and 3.6 kb upstream are readily detected after only 10 min of digestion, and the cleavage of these sites, therefore, precedes the loss of the ovalbumin coding DNA in these same hen oviduct nuclei (Figure 5). The time course of the appearance of the cutting sites at positions 5.4, 3.1, 1.5, and 1.3 kb upstream, in contrast, is more gradual and appears to be slower than the time course of the selective cleavage of the ovalbumin coding sequences (Figures 5 and 7). The major endogenous nuclease cutting sites were also detected in oviduct nuclei obtained from hormone-stimulated and -withdrawn chicks (Figure 8B). Since these discrete sites were found in oviduct chromatin from estrogen-withdrawn chicks, it is apparent that their presence can precede the transcriptional activation of the ovalbumin coding DNA by steroid hormones. As expected from the results presented in Figure 8A, the minor endogenous nuclease cutting sites at 1.3 and 1.5 kb upstream from the ovalbumin gene in the hen oviduct were detected also when *Ava*II, instead of *Eco*RI, was employed in the analysis (Figure 8C).

Discussion

Previous studies have shown that the dramatic effects of steroid hormones on the expression of the ovalbumin gene in

the chick oviduct were correlated with an alteration in a nuclease-sensitive feature of the ovalbumin chromatin template (Bloom & Anderson, 1979, 1982; Vanderbilt et al., 1982). This chromatin conformation was defined experimentally by its preferential sensitivity to minimal digestion with micrococcal nuclease and a nuclease endogenous to oviduct nuclei. The results of this study provide additional support for the proposal that the degree of sensitivity of this chromatin conformation to micrococcal nuclease is related to the transcriptional rate in the oviduct system since the rapidly transcribed ovalbumin gene in hen oviduct nuclei was cleaved preferentially by this enzyme whereas the modestly transcribed X and Y genes were not (Figure 3, Table I). Moreover, the present results confirm that this conformation is different from the chromosomal state attacked during the extensive digestion of oviduct nuclei with DNase I since the latter is apparently independent of transcriptional rate (Garel et al., 1977; Lawson et al., 1980), encompasses the coding regions of all of the members of the ovalbumin gene family and their flanking sequences (Lawson et al., 1980, 1982), and is retained after hormone withdrawal when transcriptional inactivation of the ovalbumin gene family occurs (Shepherd et al., 1980; Lawson et al., 1982). Since the micrococcal nuclease sensitive feature of the ovalbumin chromatin is probably not a direct consequence of the transcription process per se (Bloom & Anderson, 1982), it is likely that the sensitive conformation plays some role in transcriptional regulation in the oviduct.

The chromatin feature attacked selectively by micrococcal nuclease has been correlated with transcriptional activity in a variety of gene systems. These systems include the ovalbumin gene in the hormone-treated oviduct (Bloom & Anderson, 1979, 1982), the globin genes in immature avian erythrocytes (Bloom & Anderson, 1979), the heat-shock genes in heat-stressed *Drosophila* cells (Wu et al., 1979; Levy & Noll, 1981; Levinger & Varshavsky, 1982), and the active ribosomal genes in *Xenopus* and *Physarum* (Reeves, 1978; Johnson et al., 1979). The endogenous nuclease also differentiates between transcriptionally active and inactive states of the ovalbumin gene in the chick oviduct (Vanderbilt et al., 1982; Figures 6 and 7), but it is uncertain whether this property is peculiar to the oviduct or representative of other gene systems. In this regard, the globin genes in the mature erythrocyte, which have been transcriptionally inactivated during erythroid development, are apparently more rapidly autodigested than the globin genes in the oviduct, which have never been expressed (Figure 5). Whether the transcribing globin chromatin in immature erythroid cells is even more sensitive to the endogenous nuclease than the inactivated globin chromatin in the mature erythrocyte is not known at this time.

It has been established that the boundaries of the transcriptional unit of the ovalbumin gene are roughly coincident with those sequences coding for the 5′ and 3′ ends of the mature ovalbumin mRNA (Roop et al., 1980; Tsai et al., 1980). The results presented in Figures 3, 5, and 6 suggest that the entire ovalbumin transcriptional unit is packaged into a chromosomal state that is preferentially sensitive to micrococcal nuclease and to the endogenous nuclease. The chromatin boundaries at the 3′ (Table I, Figures 5 and 7) and 5′ (Table I, Figure 5) ends of the transcriptional unit, which have also been observed in other gene systems, may play an important role in delineating the transcriptional unit from flanking sequences in chromatin (Flint & Weintraub, 1977; Johnson et al., 1979; Levy & Noll, 1981; Weintraub et al., 1981). For example, the hexanucleotide AAUAAA in the 3′-untranslated region of eukaryotic mRNAs is thought to be a signal for transcription termination or polyadenylation (Proudfoot & Brownlee, 1976). This sequence, however, occurs 7 additional times within the natural ovalbumin gene (Woo et al., 1981). Since these internal sites are not used for the termination of transcription or polyadenylation in vivo (Roop et al., 1980; Tsai et al., 1980), the specificity of these biological functions must not reside within this sequence alone (Woo et al., 1981). It is conceivable that the transition in chromatin structure in the vicinity of the 3′ end of the ovalbumin gene observed in the present studies plays a role in these processes.

The property of the transcribed ovalbumin chromatin that is recognized by micrococcal nuclease and by the nuclease endogenous to oviduct nuclei is not yet known. Active genes are usually undermethylated at CpG dinucleotides [for a review, see Ehrlich & Wang (1981)], and correlations between the chromatin regions that are undermethylated, regions that are DNase I sensitive, and regions that are transcribed have been reported (Kuo et al., 1979; Groudine et al., 1981; Weintraub et al., 1981). It is unlikely, however, that DNA undermethylation is directly related to the nuclease-sensitive feature of the transcription unit of the ovalbumin gene reported in this study. Although the ovalbumin gene in the hen oviduct is unmethylated at many sites, the undermethylation extends beyond the coding region into transcriptionally inactive flanking sequences in both 5′ and 3′ directions (Mandel & Chambon, 1979). The ovalbumin gene region in the oviduct also retains its undermethylated pattern following estrogen withdrawal (Mandel & Chambon, 1979). In addition, DNA replication is thought to be essential for changing patterns of DNA methylation (Burdon & Adams, 1969; Bird, 1978) whereas the synthesis of DNA is apparently not required for the rapid activation of ovalbumin gene transcription by steroid hormones (Swaneck et al., 1979). Thus, the undermethylation of the ovalbumin gene region in oviduct chromatin, like its sensitivity to DNase I, is related to the developmental potential of the locus (Mandel & Chambon, 1979), in agreement with the proposal that variations in DNA methylations represent an important feature in the commitment of a cell to express a given gene (Holliday & Pugh, 1975; Riggs, 1975).

Studies by Wu (1980) have demonstrated that specific chromatin sites upstream from the 5′ side of several heat-shock genes in *Drosophila* are exquisitely sensitive to attack by DNase I. Hypersensitive DNase I sites upstream from active chicken globin genes (Weintraub et al., 1981), rat insulin genes (Wu & Gilbert, 1981), mouse immunoglobulin Cμ genes (Storb et al., 1981), and other eukaryotic genes [reviewed in Elgin (1981)] have also been described. Since the chromatin sites adjacent to the heat-shock genes are present prior to transcriptional activation of these genes by heat treatment, it was proposed that these regions may play a role in determining the potential for gene expression (Wu, 1980). The results in Figure 8 show that the endogenous nuclease also introduces several site-specific DNA cleavages upstream from the transcribed as well as the inactive ovalbumin gene in oviduct but not erythrocyte nuclei incubated in vitro. The apparent tissue specificity of these nuclease cutting sites indicates that they result either from unique chromatin conformations in the region 5′ to the ovalbumin gene in oviduct chromatin or from the masking of sequence-specific nuclease cutting sites in the chromatins from nonoviduct sources. Digestion of naked oviduct and erythrocyte DNAs with the purified endogenous nuclease is required to distinguish between these possibilities. This analysis would be difficult, however, because a large number of DNases have been purified from

eukaryotic nuclei (Ishida et al., 1974; Fischman et al., 1979; Machray & Bonner, 1981), and it is uncertain which, if any, of these enzymes is responsible for the results presented in Figure 8. Our preliminary studies indicate that the region containing the endogenous nuclease cutting sites is quite extensive with a range that spans from at least 1.3 to 8.8 kb upstream from the 5′ end of the ovalbumin gene. Although the region immediately adjacent to the gene on the 5′ side was not particularly sensitive to minimal micrococcal and endogenous nuclease digestions (Table I, Figure 5), a recent report that employed relatively extensive digestions by micrococcal nuclease has revealed that this region lacks the typical nucleosomal arrangement that is characteristic of the bulk of the chromatin DNA (Bellard et al., 1982). It is likely, therefore, that the large patch of chromatin upstream from the ovalbumin gene possesses a number of unique structural features that are recognized by micrococcal nuclease and the nuclease endogenous to oviduct nuclei. Whether specific chromatin sites are ever cleaved in vivo by the nuclease endogenous to eukaryotic nuclei represents an important problem for future investigation. In this regard, chromatin sites upstream from the immunoglobulin Cμ gene in lymphocytes are hypersensitive to DNase I cleavage, and the cleavage of these sites has been implicated in the in vivo rearrangement of the immunoglobulin genes (Storb et al., 1981).

The results presented in this paper and in the references discussed above can be considered in terms of one popular model for chromosome structure. Several features of the model are consistent with observations made on the heat-shock genes in *Drosophila* chromatin (Wu et al., 1979; Wu, 1980; Levy & Noll, 1981; Levinger & Varshavsky, 1982) and the globin genes in the chromatin from chicken erythroid cells (Stalder et al., 1980a,b; Weintraub et al., 1981). In the oviduct from the hen or estrogen-stimulated chick, the ovalbumin and related X and Y genes exist within the framework of a large DNase I sensitive chromatin domain consisting of greater than 100 kb of DNA (Lawson et al., 1980, 1982). Within this larger domain is at least one subdomain of chromatin structure that is operationally defined as that region selectively attacked by micrococcal nuclease and the nuclease endogenous to oviduct nuclei. This subdomain corresponds to the entire ovalbumin coding DNA (Table I and Figures 3 and 5–7) and a series of specific sites on the 5′ side of this DNA (Bellard et al., 1982; Figure 8). The chromatin boundaries of the coding region at the 3′ and 5′ ends of the ovalbumin gene appear discrete and may therefore represent important features in delineating the transcriptional unit of the ovalbumin gene in the oviduct. Upon the transcriptional inactivation of the ovalbumin gene after estrogen withdrawal, the hypersensitive sites flanking the ovalbumin gene (Figure 8) and the large DNase I sensitive domain (Lawson et al., 1982) are retained in the oviduct chromatin, while the coding region of the ovalbumin gene is rendered insensitive to both micrococcal nuclease and the endogenous nuclease (Bloom & Anderson, 1979, 1982; Vanderbilt et al., 1982; Figures 6 and 7). The hypersensitive sites flanking the ovalbumin gene, as well as the DNase I sensitive region surrounding the ovalbumin gene family, are present, therefore, in cells that possess the potential for activating the ovalbumin gene family by steroid hormones. It will be of interest to determine whether there are specific nuclease cutting sites upstream from other inducible genes in the oviduct and whether there is a relationship between the relative location, number, and nuclease sensitivity of such sites and the potential for transcriptional activation of the downstream coding regions.

Acknowledgments

We thank Bonnie Germain for her skillful technical assistance and Connie Philbrook for typing the manuscript.

Registry No. DNase I, 9003-98-9; micrococcal nuclease, 9013-53-0.

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H1 Histone Kinases from Nuclei of *Physarum polycephalum*†

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ABSTRACT: Nuclear H1 histone kinase activity from *Physarum* plasmodia was separated into three major components by DEAE-cellulose chromatography. The enzyme fractions were termed kinase R, kinase A, and kinase B and were characterized by investigating (i) their dependence on cAMP, (ii) the effect of the heat-stable inhibitor of cAMP-dependent protein kinase, (iii) the sites in calf H1 histone phosphorylated by each kinase, and (iv) the location of incorporation of phosphate in *Physarum* H1 histone catalyzed by each kinase in vitro. All three kinases were unaffected by the addition of 1 μM cAMP to the assay mixture although inhibition of the activities of kinase A and kinase B was observed in the presence of the protein kinase inhibitor. Kinase A phosphorylated mainly serine-37 in calf H1, and we conclude that the enzyme is analogous to the catalytic subunit of cAMP-dependent protein kinase. Kinase B phosphorylated multiple sites mainly in the N-terminal half of calf H1 but was distinct from kinase A in its phosphorylation of *Physarum* H1. It is not known whether there exists a mammalian equivalent of kinase B. Kinase R was unaffected by the protein kinase inhibitor and phosphorylated multiple sites in both the N-terminal and C-terminal halves of calf H1. These sites included those identified as being phosphorylated by mammalian growth-associated H1 histone kinase (kinase GR), suggesting that kinase R is analogous to mammalian kinase GR. The identification of kinase GR in *Physarum* nuclei indicates an evolutionary stability of both enzyme specificity and the structures surrounding growth-associated phosphorylation sites in H1 histones. Conservation of these features suggests that the multiple phosphorylation reactions occurring on H1 histones are essential to mechanisms which modulate chromatin structure. We also report that *Physarum* H1 is digested by chymotrypsin in an analogous fashion to calf H1.

Early studies of H1[1] histone phosphorylation during the naturally synchronous cell cycle in *Physarum polycephalum* demonstrated a large increase in H1 phosphate content during the progression from late G2 phase to mitosis (Bradbury et al., 1973). Bradbury et al. proposed that this modification of H1 was involved in the initiation of chromosome condensation. Extensive phosphorylation of H1 has also been observed in artificially synchronized mammalian cell systems, including CHO (Gurley et al., 1973, 1978), rat hepatoma (Langan et al., 1980), and HeLa (Ajiro et al., 1981), suggesting that H1 phosphorylation is a universally occurring process directly involved in chromosome condensation and

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[1] Abbreviations: H1, histone 1; CHO, chinese hamster ovary; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Cl_3CCOOH, trichloroacetic acid; cAMP, adenosine cyclic 3′,5′-phosphate; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/83/0422-0030$01.50/0